Improved Cleaning Methods for Planetary Protection Bioburden Reduction

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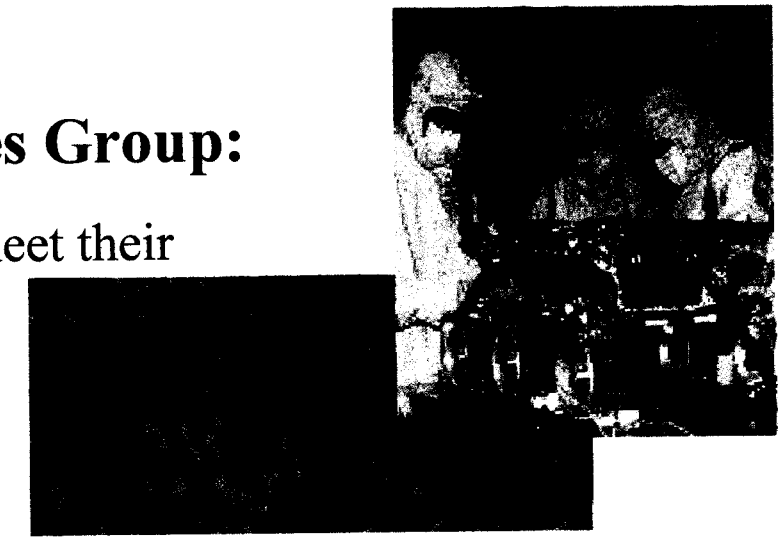
Introduction



JPL Planetary Protection Technologies Group:

- Develop technologies to enable missions to meet their planetary protection requirements.
- Support science objectives of life detection missions.

Planetary Protection Technologies:

- **Clean** - remove biological contamination, i.e. particles, viable/nonviable organisms and residues from hardware.
- **Sterilize** - eliminate viable organisms on hardware.
- **Validate** - determine hardware biological decontamination effectiveness by quantifying and identifying remaining biological contamination.
- **Archive** - record biological history of hardware materials, assembly area, spacecraft, and launch site against future PP and science requirements.
- **Maintain** – develop cross contamination modeling tools and develop methods to identify/minimize/ remove any cross contamination or recontamination during assembly, transit, functional hardware test, rework, landing and any Earth return.

Cleaning Technologies:

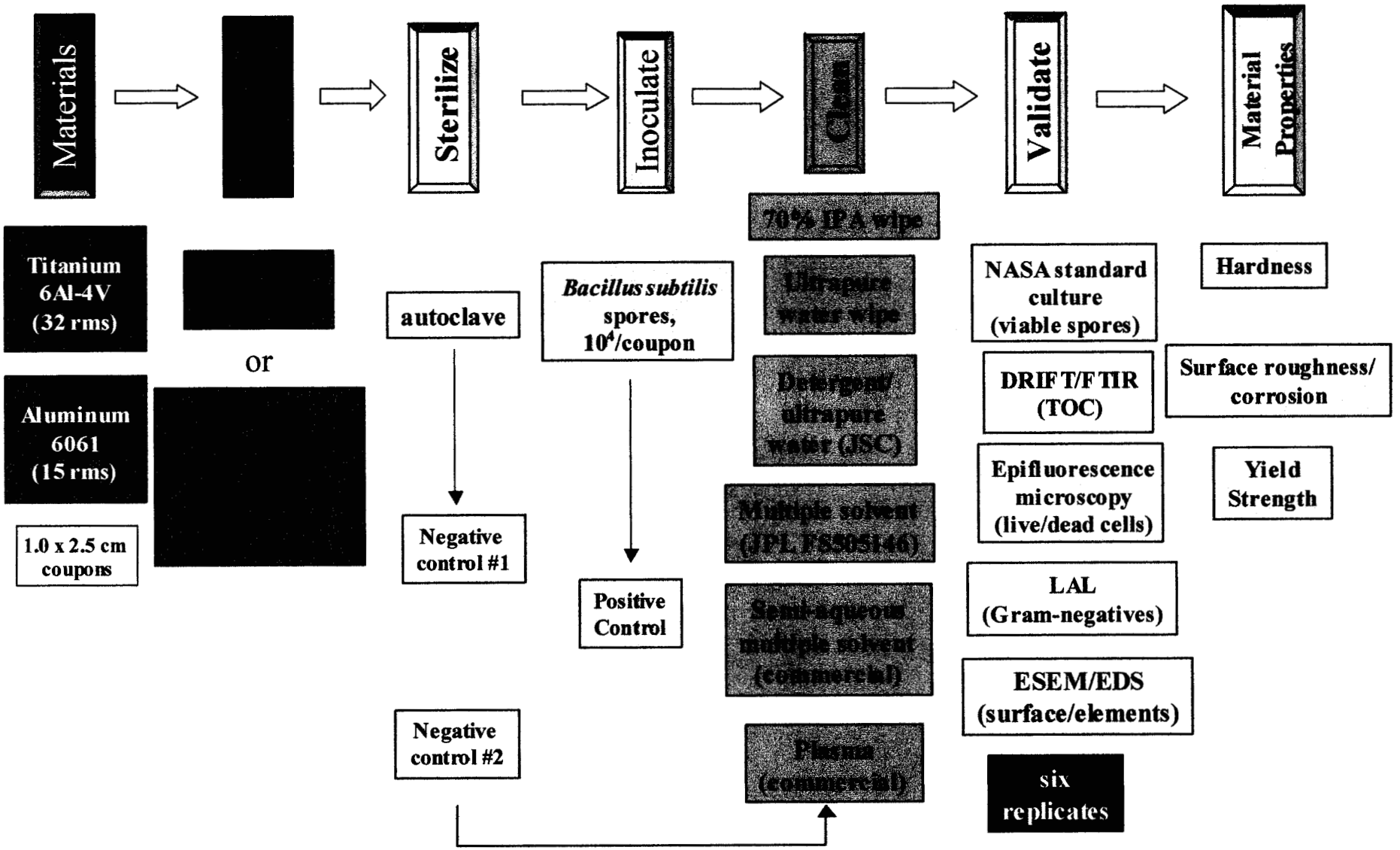
- Designed a comprehensive matrix to study the effectiveness of a variety of methods for biologically cleaning space flight hardware materials, i.e.
  - sample handling or in-situ experiment hardware materials
  - general (bulk) hardware materials

- Matrix consists of coupons of hardware materials (identified by Mars Sample Return Project) inoculated with *Bacillus subtilis* spores.

- *Bacillus subtilis* spores are NASA "standard bug," are hardy and difficult to clean

- Coupons were then cleaned using a variety of cleaning methods and the degree of biological cleanliness is measured using a suite of techniques.

Cleaning Matrix



Experimental

Materials

1.0 x 2.5 cm coupons

- **Aluminum, Al 6061 T6** (mill finish 15 rms) & mirror polished (2 rms, limited matrix)
  (0.65 Si, 0.44 Fe, 0.27 Cu, 0.02 Mn, 0.96 Mg, 0.20 Cr, 0.02 Ti)
- **Titanium, Ti 6Al 4V** (mill finish 32 rms)
  (0.02 C, 0.152 O, 0.15 Fe, 0.015 N, 6.4 Al, 3.9 V)

[redacted]

- coupons placed, in a single layer, into pyrex petri dishes
- covered petri dishes were placed in steam autoclave bags and sterilized at 121°C for 15 minutes, followed by a 30-minute drying cycle

Sterilization

- clean room polyester wipes saturated with acetone to remove residual adhesive;
- freon vapor degreased for one hour then rinsed with isopropyl alcohol and dried

Negative Control #1 - no further treatment

Negative Control #2 - cleaned, but NOT inoculated

Inoculation

- coupons were inoculated with 100 µl water suspension of 5.8×10^3 culturable (1.4×10^4 total) *Bacillus subtilis* spores in water and allowed to dry in air

Positive Control - inoculated, but NOT cleaned

Cleaning Methods

70% Isopropyl Alcohol (IPA) Wipe

- recommended JPL cleaning method
- mechanically wiped with 9"x9" clean room polyester wipes (Coventry 6209 c-prime, freon washed) saturated with a 70% v/v IPA/ultrapure water; performed in Class 100 biohazard hood or Class 100 laminar flow bench wearing powder-free nitrile gloves

Ultrapure Water (UPW) Wipe

- previous studies found that some vegetative cells are better removed with pure water than with 70% IPA
- mechanically wiped with 9"x9" clean room polyester wipes (Coventry 6209 c-prime, freon washed) saturated with certified Sigma (Cat. No. W-4502) 18 MΩ water; performed in Class 100 biohazard hood or Class 100 laminar flow bench wearing powder-free nitrile gloves

Multiple Solvent

- JPL Manufacturing Process Specifications FS505146 Rev. C recommended method for cleaning Al and Ti flight hardware materials
- involves ultrasonic cleaning with acetone and IPA, followed by alkaline cleaning with Oakite 61B with deionized water rinse and drying with clean, dry nitrogen; for Ti *only* procedure includes nitric acid passivation step after alkaline cleaning.
- procedure validated using MIL-STD 1246C (UV test at 3000-4000 nm, particle counts, molecular contamination)

Detergent/ultrapure water (UPW)

- employed by Lockheed Martin/JSC Curation Team
- rub surfaces with dilute Joy detergent using soft, polyester knit cloth and rinse with UPW; repeat rub/rinse 3 times
- agitate coupons in nitrogen-agitated UPW bath at 75°C for 30 minutes; then air dry (d

Semi-aqueous, multiple solvent

- commercially available, integrated cleaning method
- same method used for both Al and Ti
- acid cleaner for removing bacteria followed by deoxygenated metal cleaning solution then rinse with 18 MΩ, degassed, deionized water and dried with hot, clean nitrogen at 74-82°C

Plasma cleaning

- commercially available, oxygen-plasma cleaning method

Validation

NASA Standard Cultures

- as per NASA Procedure for the Microbial Examination of Space Hardware (NPG: 5340.1C)

Three culturing procedures performed:

- Sterility assay: culture entire coupon in (TSB, tryptic soy broth) → detects any culturable spores remaining on coupon after cleaning
- Supernatant assay: sonicate coupon then culture supernatant on a TSA (tryptic soy agar) plate → culturable spores removed from coupon after cleaning and sonication
- Coupon assay: culture sonicated coupon on TSA plate → culturable spores NOT removed from coupon after cleaning and sonication

DRIFT/FTIR

- method used for monitoring hardware molecular contamination, per MIL-STD 1246C
- organic contamination is extracted from the surface of the coupon with dichloromethane, and the residue is evaporated onto KBr power under dry nitrogen
- performed on coupons and spore inoculant solution

Epifluorescence microscopy

- method front-lined as new flight hardware cleaning validation technique
- coupons are dipped in solution of Molecular Probe Syto-9 dye and then counted
- also washed with Triton-X, collected onto a 25mm filter and counted

Limulus Amebocyte Lysate (LAL)

- highly specific and sensitive assay for Gram-negative bacteria that makes use of the unique immune system enzyme cascade initiated in the blood cells (amebocytes) of Limulus polyphemus (horseshoe crab)
- conducted by Marine Biological Laboratory, Woods Hole

Environmental Scanning Electron Microscopy/Energy Dispersive Spectroscopy (ESEM/EDS)

- ESEM allows field-of-view down to μm level
- EDS provides elemental analysis of μm sized surface features

Materials Properties

- surface roughness/corrosion
- yield strength
- hardness

Results

Material	Positive control	70% IPA	UPW	Detergent/ UPW	Multiple solvent*	Semi-aqueous multiple solvent
Al 6061 (15 rms)	Y(1378)	Y Y(8) Y	Y Y(8) Y	Y Y(4) Y	Y Y(3) Y	Y Y(849) Y
	200	140	800	430	100	60
Ti 6Al-4V (32 rms)	Y(2950)	Y Y(106) Y	Y Y(98) Y	Y Y(19) Y	N N(1) N	N N(1) N biological remnants seen
	130	110	1500	1400	51	100

- NASA Standard Culture
  - sterility assay (Y/N)
  - supernatant assay (culture count)
  - coupon assay (Y/N)
- DRIFT/FTIR (ng/cm^2)
  - results from 6 pooled coupons
  - LOD = 50 ng/cm^2

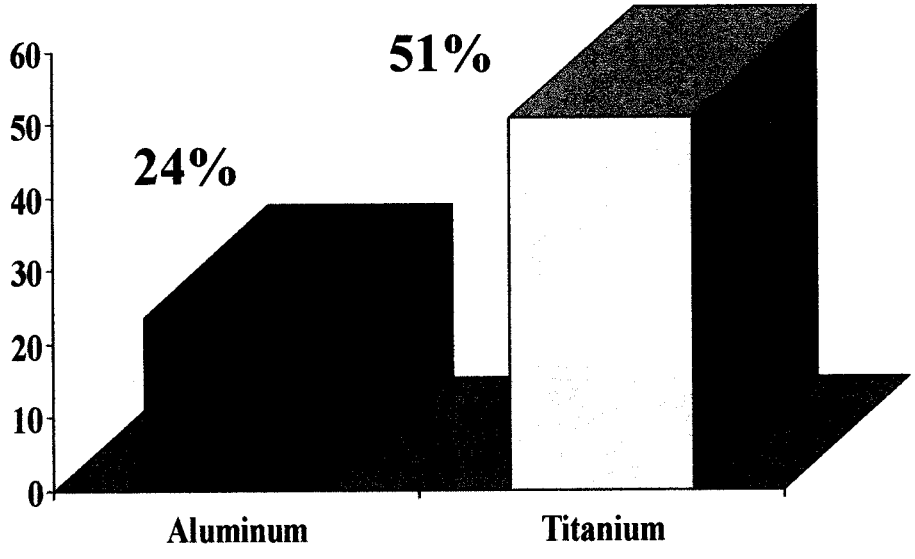
- Secondary contamination
  - Agar plate (culture count)
  - LAL (EU/coupon)

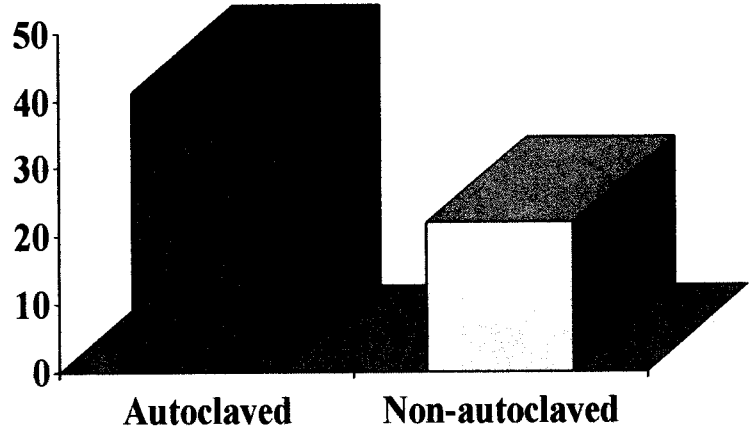
*validated per MIL-STD 1246C at particle cleanliness level of 100

non pre-cleaned positive and negative controls

IPA tested positive

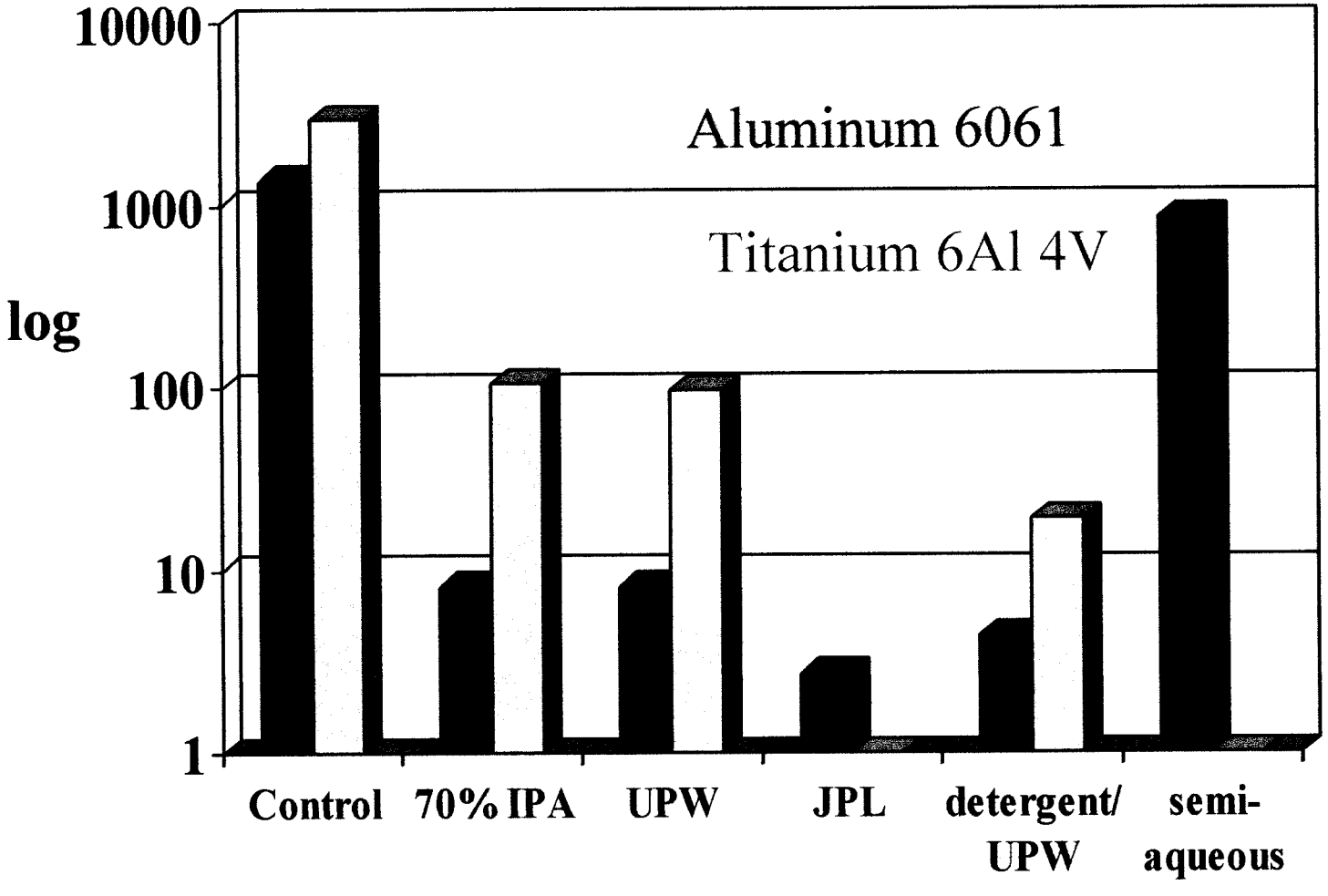
Supernatant Assay
% of recoverable culturable spores in supernatant



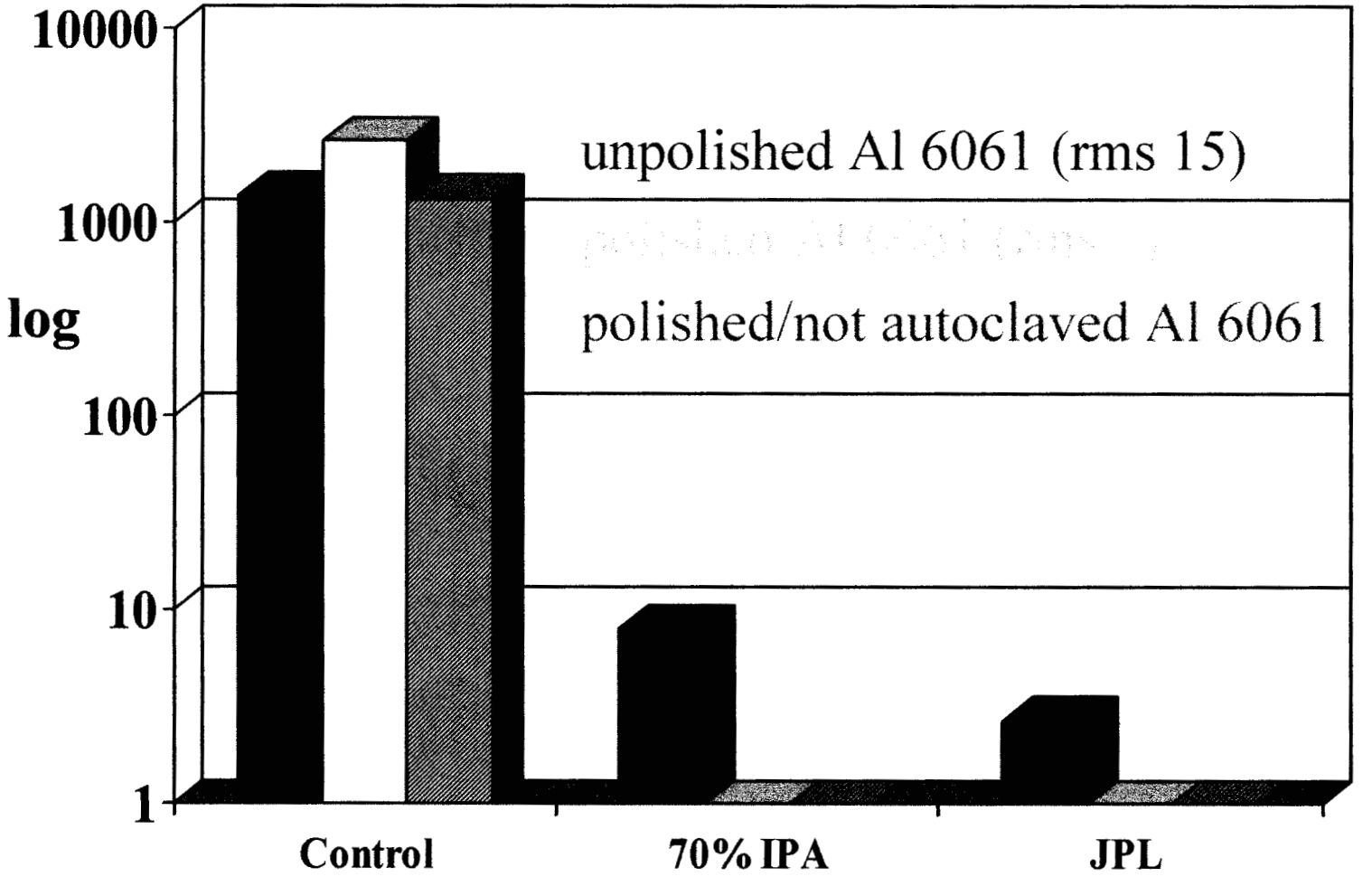


Supernatant Assay

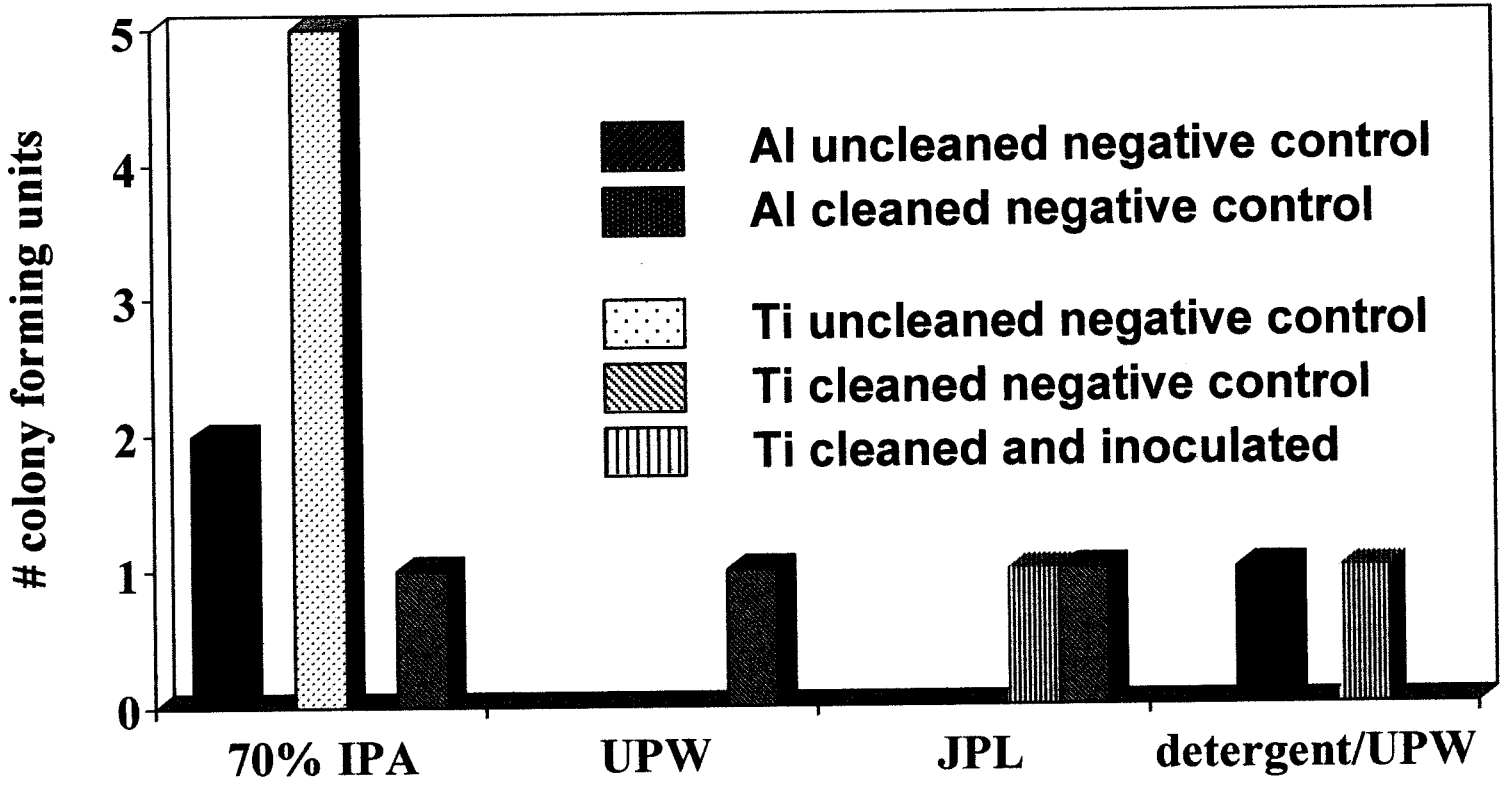
Uncorrected!!! **# of recoverable culturable spores in supernatant**



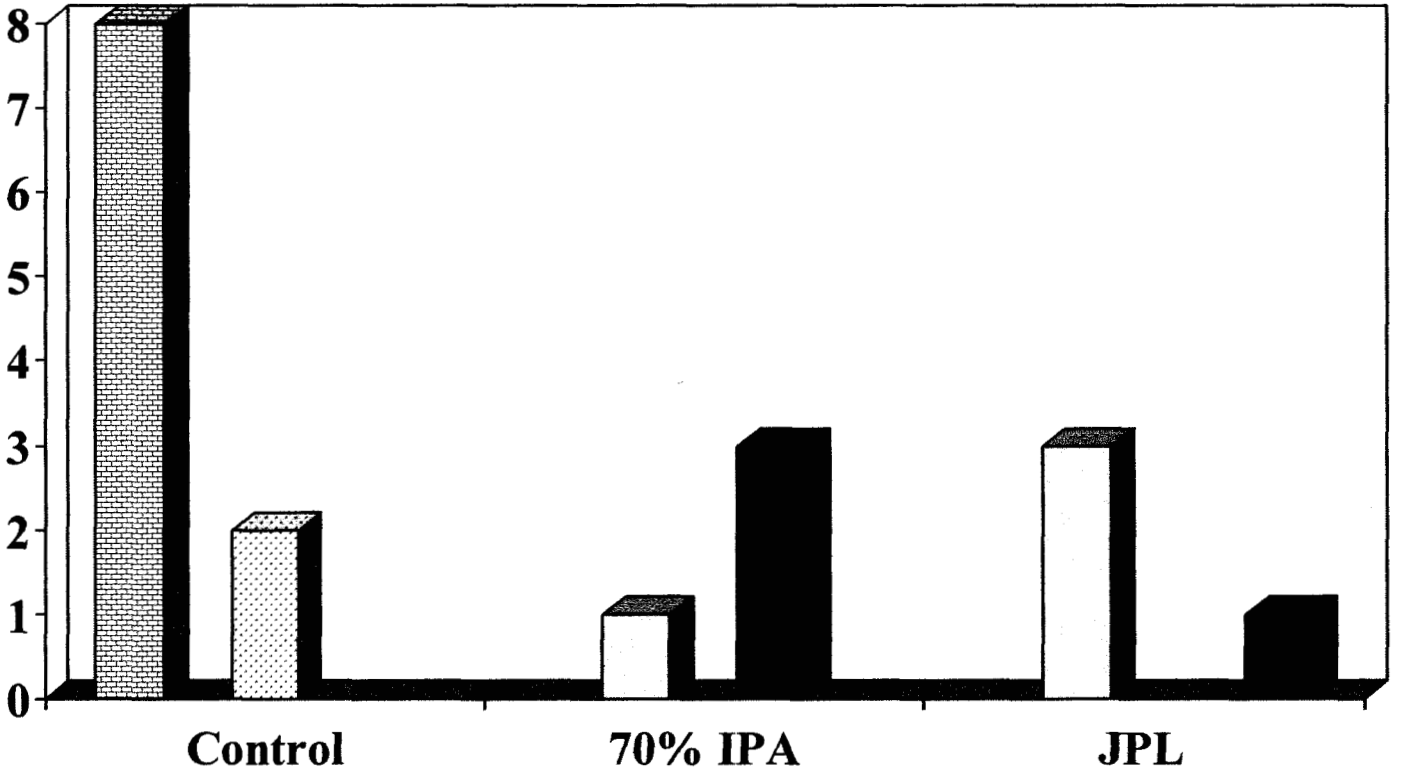
Supernatant Assay
Preliminary Results Polished Al
# of recoverable culturable spores in supernatant



Coupons with secondary contamination unpolished Al 6061 and Ti 6Al 4V



Coupons with secondary contamination polished Al 6061 (2 rms)



PA: Polished & sterilized aluminum; PUS: Polished non-sterilized aluminum

- sources of secondary biological contamination are low for unpolished Al 6061 and Ti 6Al 4V studies.

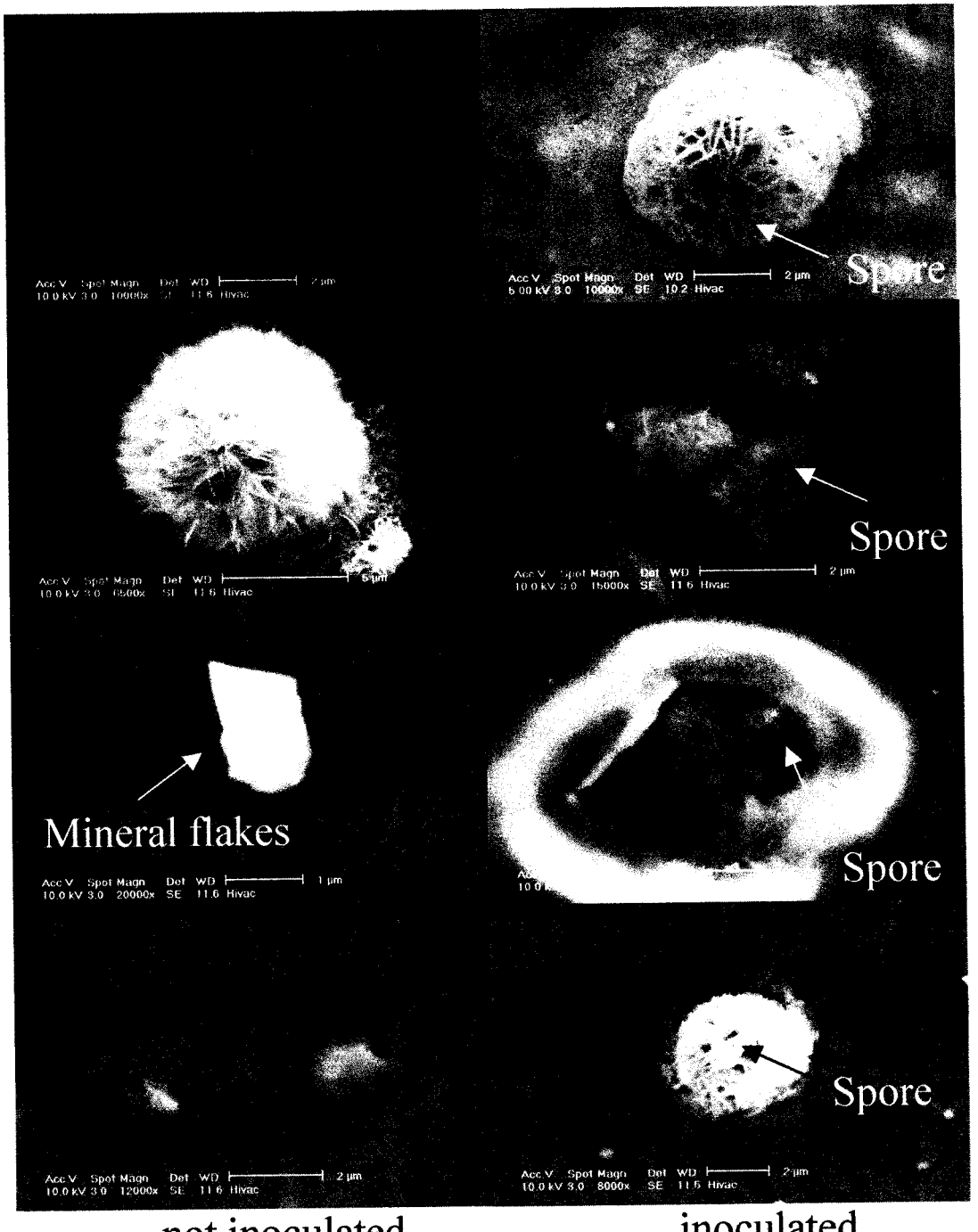
- secondary, non-biological, organic contamination measured with DRIFT/FTIR for unpolished Al 6061 and Ti 6Al 4V consists of traced aliphatic esters (commonly seen on cleaned flight hardware), silicone, organic acids, and aliphatic hydrocarbons traced to clean room wipes used, possible incomplete pre-cleaning of coupons and aluminum foil used to cover beakers.

- for polished Al 6061, secondary biological contamination also low, with LAL assays all negative, or low.

- secondary organic contamination eliminated in polished Al study, i.e. residual organic contamination found to be <50 ng/cm^2 DRIFT/FTIR limit of detection.

Aluminum 6061 unpolished (15 rms)

ESEM



control

multiple solvent

detergent/ ultrapure water

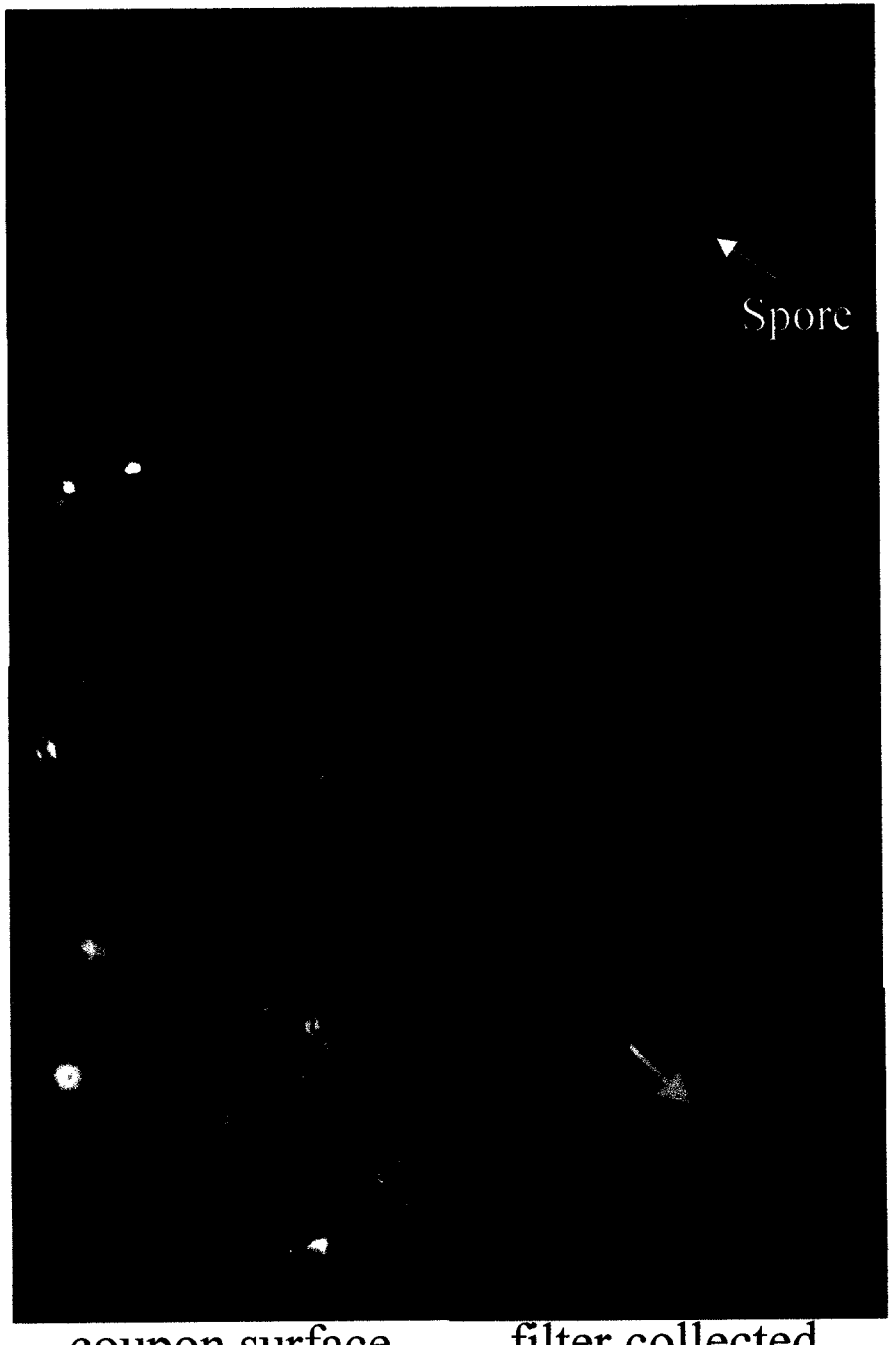
semi-aqueous multiple solvent

not inoculated

inoculated

Aluminum 6061 unpolished (15 rms)

epifluorescence microscopy



control

multiple solvent

detergent/ ultrapure water

semi-aqueous multiple solvent

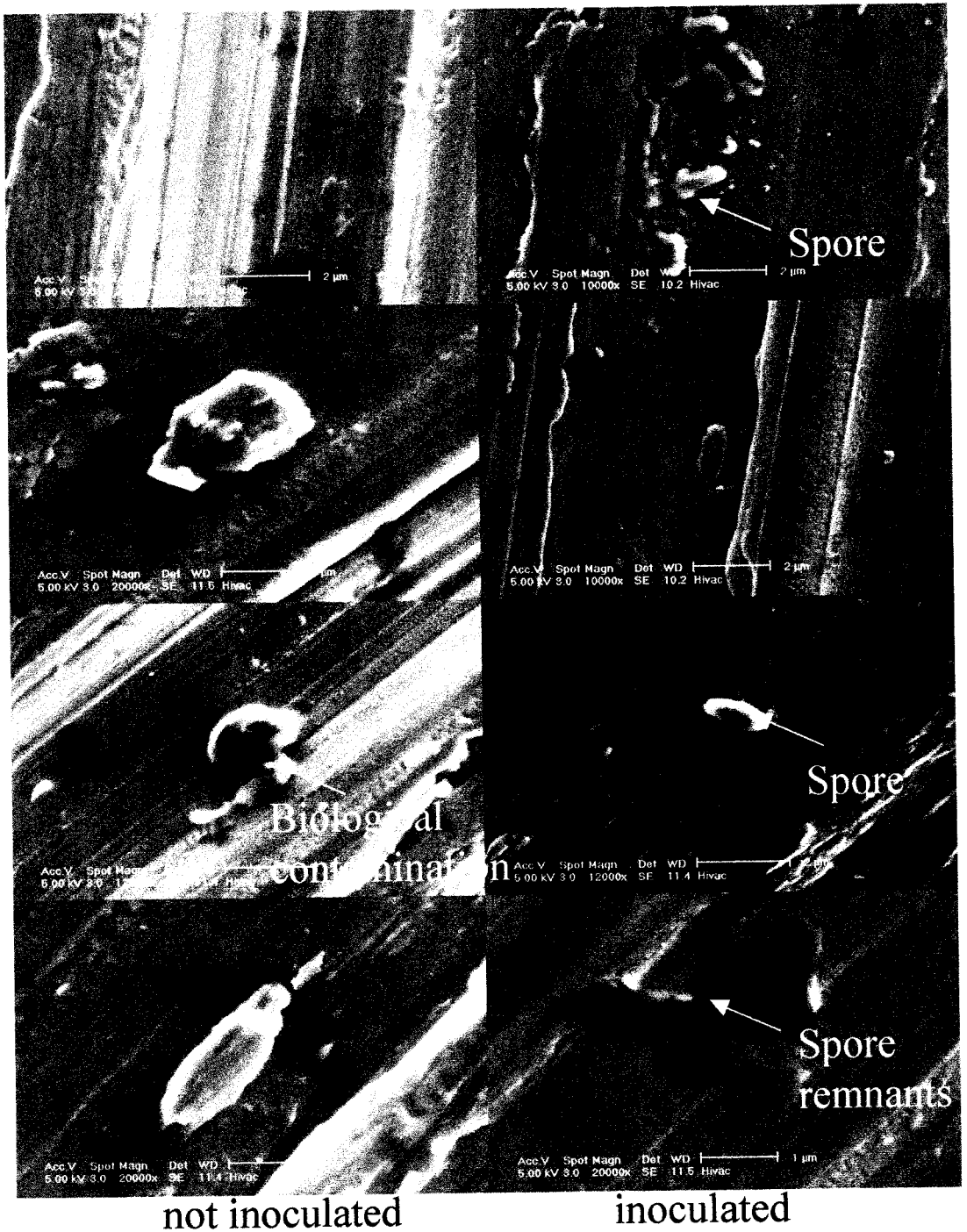
vegetative cell germinated from *B. subtilis* spores →

coupon surface

filter collected

Titanium 6Al-4V (32 rms)

ESEM



control

multiple solvent

detergent/ ultrapure water

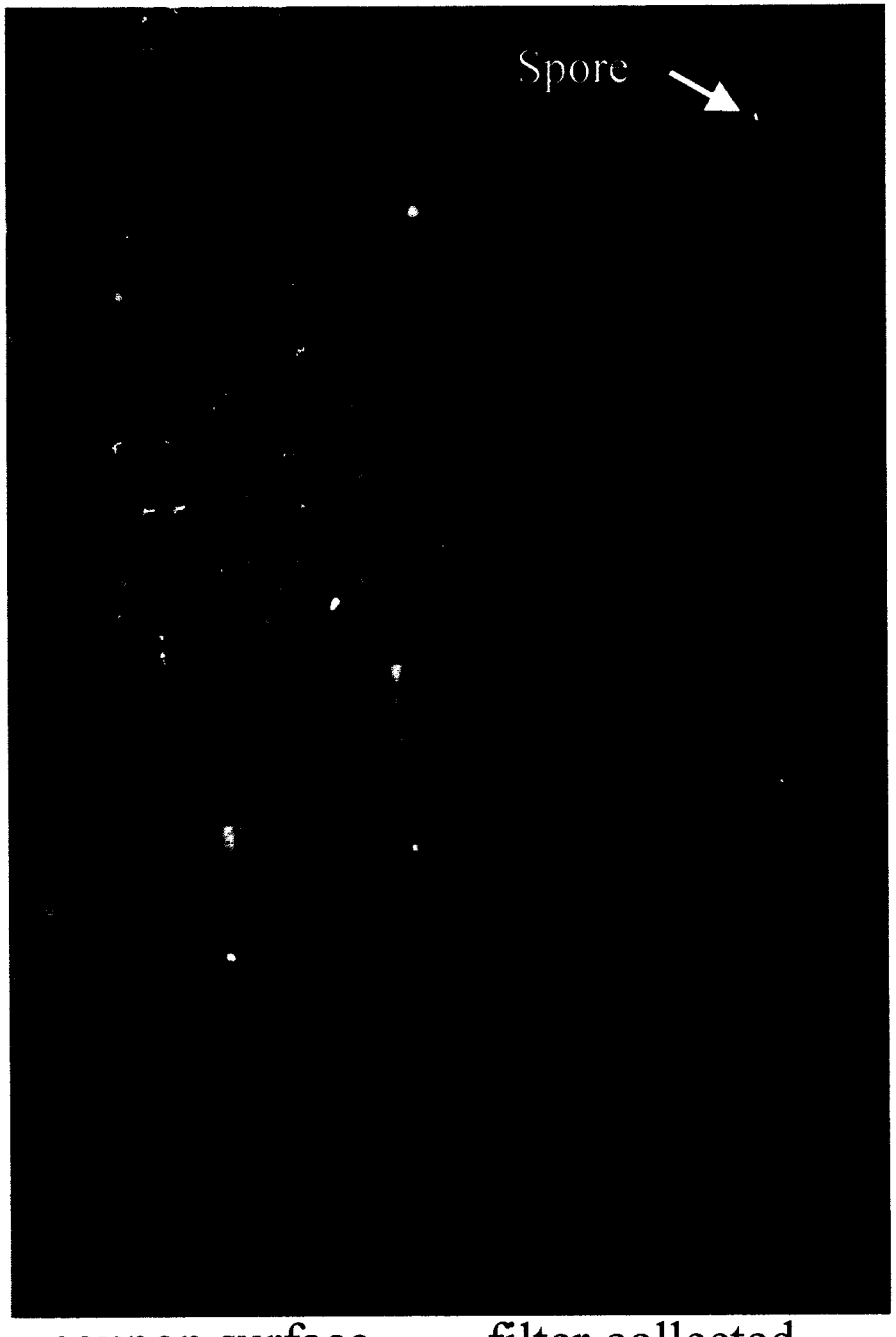
semi-aqueous multiple solvent

not inoculated

inoculated

Titanium
6Al-4V
(32 rms)

epifluorescence
microscopy



coupon surface

filter collected

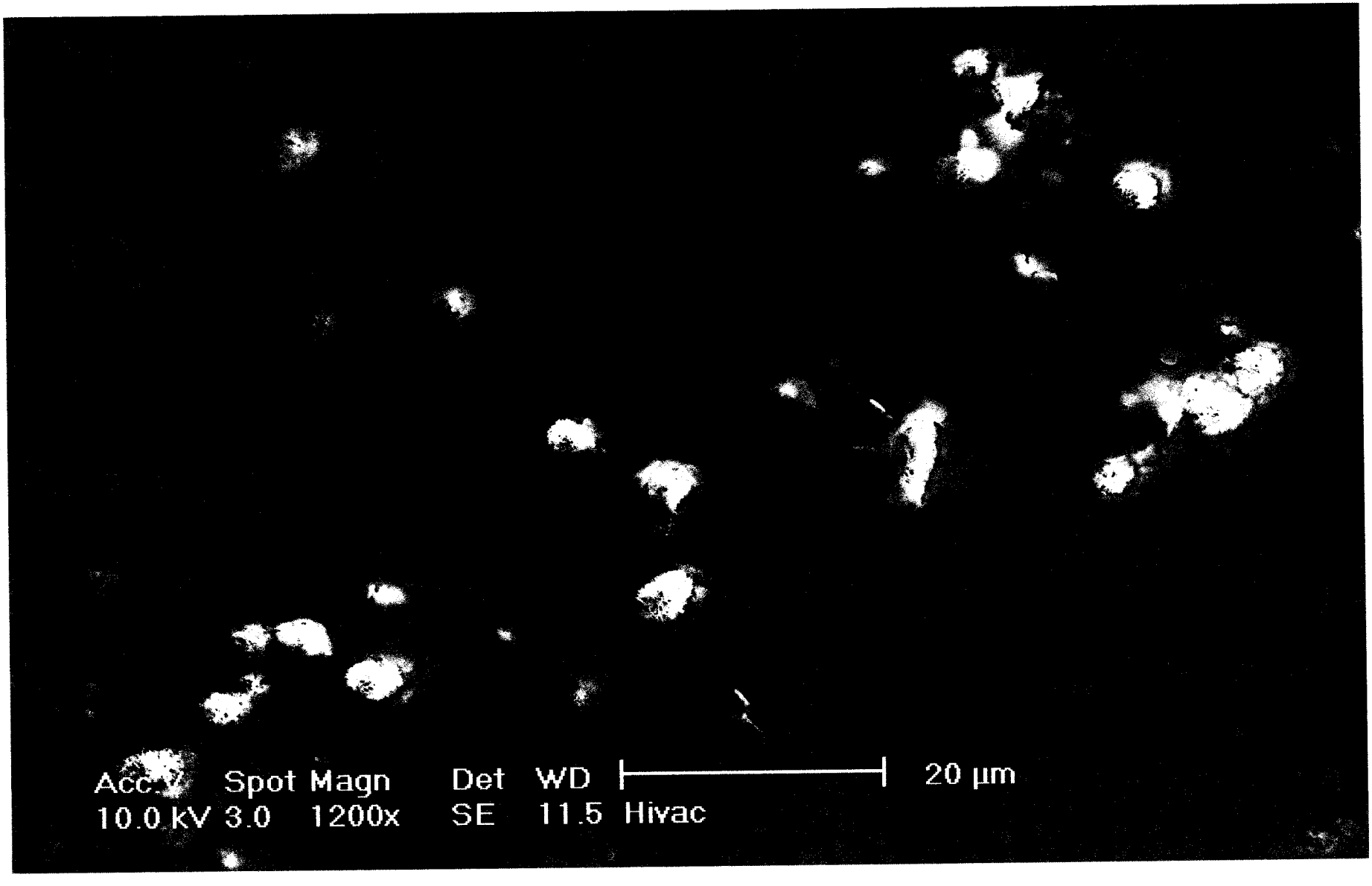
control

multiple solvent

detergent/
ultrapure water

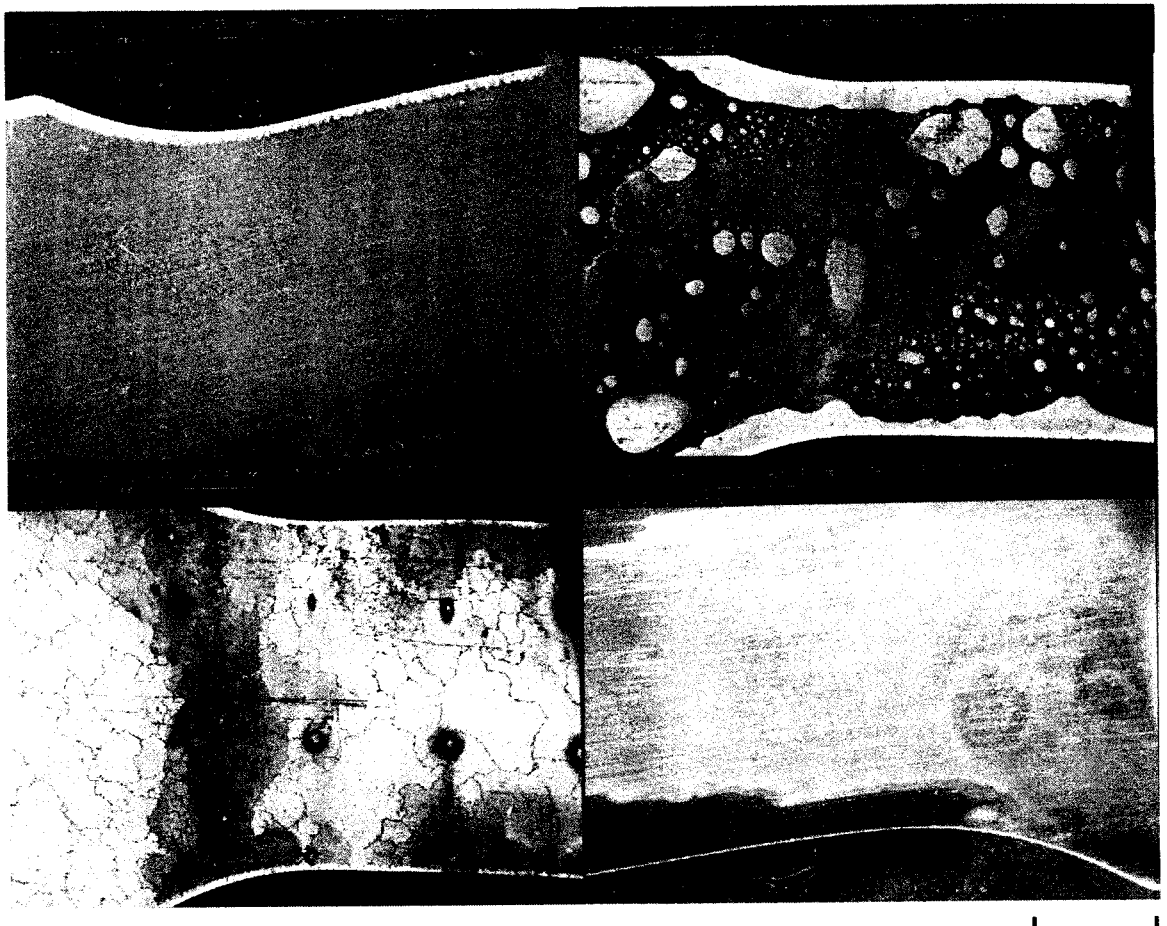
semi-aqueous
multiple solvent

Inoculated Al coupon cleaned by semi-aqueous, multiple solvent method



Surface roughness/corrosion

Al 6061
unpolished
(15 rms)
pre-cleaned



Al 6061
unpolished
(15 rms)
pre-cleaned
and autoclaved

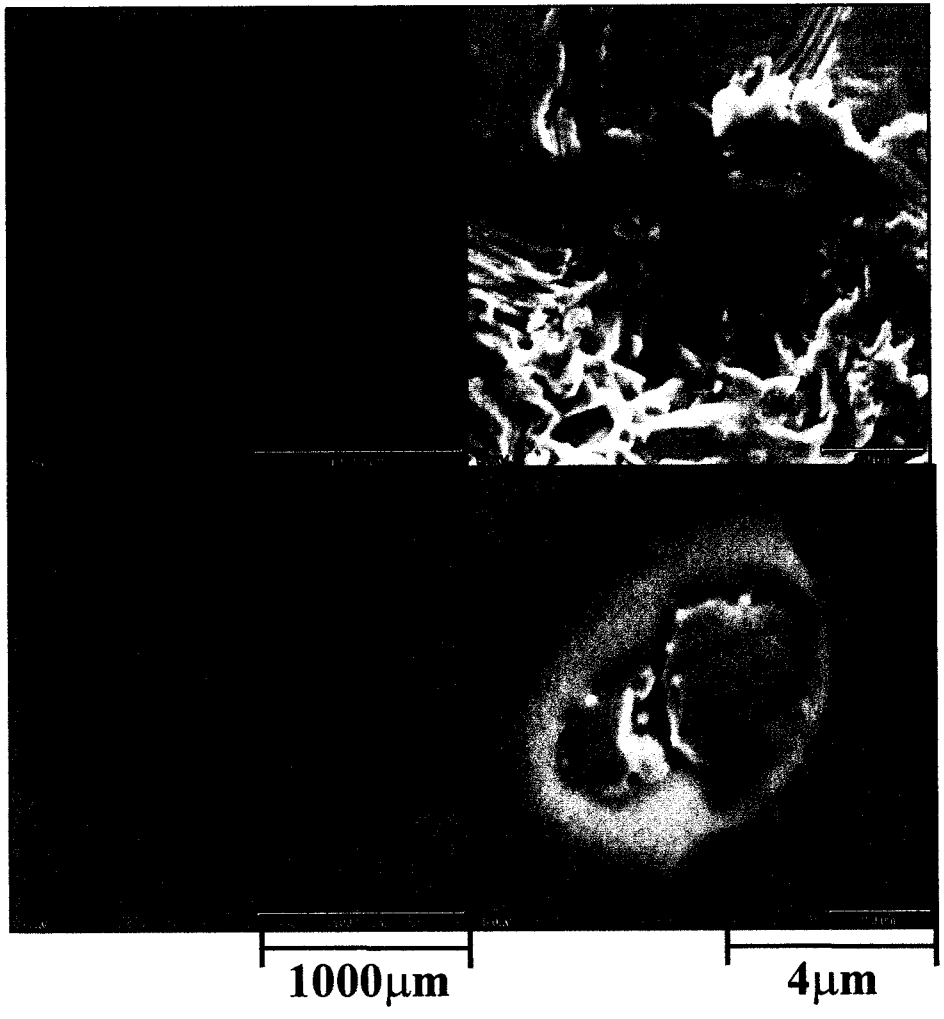
Al 6061
unpolished
(15 rms)
pre-cleaned
autoclaved and
multiple solvent
cleaned

Ti 6Al 4V
pre-cleaned,
autoclaved and
multiple solvent
cleaned

2 mm

polished Al 6061 after pre-cleaning with acetone, IPA and DCM

polished Al 6061 after pre-cleaning and autoclave sterilization



Conclusions

Cleaning matrix conducted as described using *B.subtilis* spores shows:

- all cleaning methods studied clean to some degree
- Ti 6Al-4V (mill finish 32) can be "cleaned-to-sterility" using multiple-solvent cleaning methods (JPL method FS505146 Rev.C) with *no* spore remnants and only traces of organic contamination (~50 ng/cm^2) remaining on the surface
- unpolished Al 6061 *cannot* be cleaned to sterility with any of the cleaning methods studied in the matrix
- polished Al 6061 easier to clean, but ability to "clean-to-sterility" needs to be confirmed with more replicate studies, i.e. may still be possible for spores to sit in 8 to 12μm surface defects seen on some polished Al 6061 coupons
- requisite autoclaving step leads to extensive oxidation of surface properties of hardware materials, thus results of cleaning matrix as conducted represent "worst case" cleaning scenario

- matrix leads to the formation of possible "spore houses" containing magnesium
- noted correlation between number of "spore houses" seen on coupons and vegetative growth
- number of possible "spore houses" containing magnesium found on polished Al about equal to number found on unpolished; however, size of "houses" on polished Al are smaller than those seen on unpolished Al, indicating "houses" may be composed of only inorganic materials

Future Studies

- repeat clean-to-sterility studies on Ti and polished Al to confirm robustness of multiple solvent cleaning method
- compare surface properties of autoclaved Ti and Al surfaces to those found on aged flight hardware (e.g. Mars Pathfinder and Viking mission hardware)
- conduct more detailed chemical analyses of possible "spore houses"
- extend matrix to include composite hardware materials and vegetative microbes
- modify JPL multiple solvent method to include nitric acid passivation step for Al